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(Date)

Rose M. Thiessen, Reg. No. 40,202

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to the examination of the above-identified application on the merits, please amend the application as follows:

IN THE SPECIFICATION:

Please replace the paragraph beginning at page 1, line 3 with the following rewritten paragraph:

--This application is a continuation of Application No. 09/659,470, filed on 11 September 2000, which is a continuation, under 35 U.S.C. § 120, of International Patent Application No. PCT/AU99/00152, filed on 11 March 1999, under the Patent Cooperation Treaty (PCT), which was published by the International Bureau in English on 16 September 1999, which designates

the U.S. and claims the benefit of Australian Patent Application No. PP2388, filed on 12 March 1998.--

Please insert the following paragraphs at page 4, after line 3:

--Figure 4 exemplifies the reactions taking place in a cell according to preferred embodiments.

Figure 5 illustrates the concentration profiles across an electrochemical cell according to the preferred embodiments before the application of an electrical potential, after application of the potential and prior to reaching steady state, and at steady state.

Figure 6 shows the time dependence of current prior to and after application of electrical potential.

Figure 7 shows the ferrocyanide concentration profiles across an electrochemical cell according to the preferred embodiments prior to a polarity reversal, after reversal and prior to reaching a steady state, and at steady state.

Figure 8 shows the time dependence of current prior to and after a polarity reversal.

Figure 9 shows the time dependence of current prior to and after an interruption of applied potential of 15 seconds.

Figure 10 shows the reactions in an electrochemical cell with peroxide oxidation.

Figure 11 shows the time dependence of current when an initial potential sufficient to oxidise hydrogen peroxide is applied.

Figure 12 describes the cell of Figure 10, which is suitable for use in the method of the preferred embodiments, in plan view (not to scale).

Figure 13 describes an embodiment of a cell suitable for use in the preferred embodiments in cross-section view on line 10-10 of Figure 12 (not to scale).

Figure 14 describes the cell of Figure 10, which is suitable for use in the method of the preferred embodiments, in end section view (not to scale). --

Figure 15 show the product of manufacturing Step 2 in plan.

Figure 16 shows the product of Figure 15 in side elevation.

Figure 17 shows the product of Figure 15 in end elevation.

Figure 18 shows the product of manufacturing Step 3 in plan.

Figure 19 shows the product of Figure 18 in cross-section on line 105-105 of Figure 18.

Figure 20 shows the product of manufacturing Step 5 in plan.

Figure 21 shows the product of Figure 20 in side elevation.

Figure 22 shows the product of Figure 20 in end elevation.

Figure 23 shows the product of manufacturing Step 7 in plan.

Figure 24 is a cross-section of Figure 23 on line 110-110.

Figure 25 shows the product of Figure 23 in end elevation.

Figure 26 shows a cell according to the invention in plan.

Figure 27 shows the call of Figure 26 in side elevation.

Figure 28 shows the cell of Figure 26 in end elevation.

Figure 29 shows a scrap portion of a second embodiment of the invention in enlarged section.--

Please replace the paragraph beginning at page 8, line 3 with the following rewritten paragraph:

--When using cells such as described in our co-pending applications (see, e.g., Figures 12, 13, and 14), the sample volumes are very small and heating can be achieved with low energy input.--

Please insert the following paragraph at page 8, after line 4:

--It is known to measure the concentration of a component to be analysed in an aqueous liquid sample by placing the sample into a reaction zone in an electrochemical cell comprising two electrodes having an impedance which renders them suitable for amperometric measurement. The component to be analysed is allowed to react directly with an electrode, or directly or indirectly with a redox reagent whereby to form an oxidisable (or reducible) substance in an amount corresponding to the concentration of the component to be analysed. The quantity of the oxidisable (or reducible) substance present is then estimated electrochemically. Generally

this method requires sufficient separation of the electrodes so that electrolysis products at one electrode cannot reach the other electrode and interfere with the processes at the other electrode during the period of measurement.

In PCT/AU96/00365 is described a novel method for determining the concentration of the reduced (or oxidised) form of a redox species in an electrochemical cell of the kind comprising a working electrode and a counter (or counter/reference) electrode spaced from the working electrode. The method involves applying an electrical potential difference between the electrodes, spacing the working electrode from the counter electrode so that reaction products from the counter electrode arrive at the working electrode and selecting the potential of the working electrode so that the rate of electro-oxidation of the reduced form of the species (or of electro-reduction of the oxidised form) is diffusion controlled. By determining the current as a function of time after application of the potential and prior to achievement of a steady state current and then estimating the magnitude of the steady state current, the method previously described allows the diffusion coefficient and/or the concentration of the reduced (oxidised) form of the species to be estimated.

PCT/AU96/00365 exemplifies this method with reference to use of a "thin layer" cell employing a GOD/Ferrocyanide system. As herein used, the term "thin layer electrochemical cell" refers to a cell having closely spaced electrodes such that reaction products from the counter electrode arrive at the working electrode, in practice, the separation of electrodes in such a cell for measuring glucose in blood will be less than 500 microns, and preferably less than 200 microns.

The chemistry used in the exemplified electrochemical cell is as follows:

glucose + GOD → gluconic acid + GOD* reaction 1

GOD* + 2 ferricyanide → GOD + 2 ferrocyanide reaction 2

where GOD is the enzyme glucose oxidase and GOD* is the 'activated' enzyme. Ferricyanide ($[Fe(CN)_6]^{3-}$) is the 'mediator' which returns the GOD* to its catalytic state. GOD, an enzyme catalyst, is not consumed during the reaction so long as excess mediator is present. Ferrocyanide ($[Fe(CN)_6]^{4-}$) is the product of the total reaction.

Ideally there is initially no ferrocyanide, although in practice there is often a small quantity. After reaction is complete the concentration of ferrocyanide (measured electrochemically) indicates the initial concentration of glucose. The total reaction is the sum of reactions 1 and 2:

GOD glucose + 2 ferricyanide → gluconic acid + 2 ferrocyanide reaction 3

"Glucose" refers specifically to β-D-glucose.

The prior art suffers from a number of disadvantages. Firstly, sample size required is greater than desirable. It would be generally preferable to be able to make measurements on samples of reduced volume since this in turn enables use of less invasive methods to obtain samples.

Secondly, it would be generally desirable to the accuracy of measurement and to eliminate or reduce variations due, for example, to cell asymmetry or other factors introduced during mass production of microcells.

Thirdly, it would be generally desirable to reduce the time that is required in which to obtain a measurement. The test protocols used in current commercially available electrochemical glucose sensors involve a predetermined wait period at the beginning of the test during which the enzyme reacts with the glucose to produce the specie that is sensed electrochemically. This initial period is fixed at the maximum necessary to achieve the desired reaction under all conditions of use.

Fourthly, it would be desirable to eliminate variations due to oxygen. Oxygen can be plentiful in blood, either dissolved in the plasma, or bound in hemoglobin. It can also be introduced during "finger sticking," where a blood drop of small volume and high surface area is exposed to the atmosphere prior to introduction to a cell. Oxygen can interfere since oxygen is a mediator for GOD. The reaction is as follows:

glucose + GOD → gluconic acid + GOD* reaction 4
GOD* + oxygen + water → GOD + hydrogen peroxide reaction 5

The total reaction is given by:

$$\text{glucose} + \text{water} + \text{oxygen} \xrightarrow{\text{GOD}} \text{gluconic acid} + \text{hydrogen peroxide} \quad \text{reaction 6}$$

In most situations the complication of oxygen also acting as a mediator is unwanted simply because the concentration of final ferrocyanide no longer is directly proportional to the concentration of initial glucose. Instead, the initial glucose concentration is then related to both the final concentration of ferrocyanide and of hydrogen peroxide.

A method is provided for determining the concentration of a reduced (or oxidised) form of a redox species in an electrochemical cell of the kind comprising a working electrode and a counter electrode spaced from the working electrode by a predetermined distance, said method comprising the steps of:

(a) applying an electric potential between the electrodes, wherein the electrodes are spaced so that reaction products from the counter electrode arrive at the such that the rate of the electro-oxidation of the reduced form (or oxidised form) of the redox species is diffusion controlled,

(b) determining current as a function of time after application of the potential and prior to achievement of a steady state current,

(c) estimating the magnitude of the steady state current,

(d) interrupting, or reversing the polarity, of the potential,

(e) repeating step (b) and step (c).

It was discovered that if the polarity is reversed (i.e., the anode becomes the cathode and vice versa) after the initial steady state current is achieved, then a second transient current can be observed and after a period of time a second steady state is achieved. This has proved useful for diagnosing, and for reducing the effects of, cell asymmetry and other factors which influence the transient current. It also permits greater reliability and/or accuracy of estimation by allowing measurements to be made repetitively using reverse polarities. Likewise, if the potential is interrupted for a time sufficient for the concentration profile to relax to a random state and is then reapplied, steps (b) and (c) can be repeated.

A method is provided for measuring the concentration of glucose in a sample by means of a cell having a working electrode, a counter electrode, an enzyme catalyst, and a redox mediator,

comprising the steps of operating the cell at a potential higher than that of the redox reaction so as to oxidise hydrogen peroxide at the anode and then conducting a method as described above.

By this means the interference of oxygen can be ameliorated as explained in more detail hereinafter.

A method is provided wherein the sample is allowed to react with an enzyme catalyst and a redox mediator comprising the steps of:

(a) applying a potential between the electrodes before or during filling of the cell,

(b) measuring the increase in current as a function of time,

(c) determining or predicting from the measurement in step (b) the time of completion of reaction with said catalyst, and

(d) then interrupting or reversing the polarity of the potential.

An electrochemical cell suitable for use in preferred embodiments is depicted schematically (not to scale) in Figures 12, 13, and 14. The cell comprises a polyester core **4** approximately 18 mm x 5 mm and 100 micron thick and having a circular aperture **8** of 3.4 mm diameter. Aperture **8** defines a cylindrical cell side wall **10**. Adhered to one side of core **4** is a polyester sheet **1** having a sputter coating of palladium **2**. The sputter coating took place at between 4 and 6 millibar pressure in an atmosphere of argon gas to give a uniform coating thickness of 100-1000 angstroms. The sheet is adhered by means of an adhesive **3** to core **4** with palladium **2** adjacent core **4** and covering aperture **8**.

A second polyester sheet **7** having a second sputter coating of palladium **6** is adhered by means of contact adhesive **5** to the other side of core **4** and covering aperture **8**. There is thereby defined a cell having cylindrical side wall **10** and closed each end by palladium metal. The assembly is notched at **9** to provide for a solution to be admitted to the cell or to be drawn in by wicking or capillary action and to allow air to escape.

The metal films **2**, **6** are connected with suitable electrical connections or formations whereby potentials may be applied and currently measured. The cell is furnished with GOD and ferrocyanide in dry form. In use according to the method a drop of blood is drawn into the cell at **9** by capillary action and allowed to react.

The construction of a thin layer electrochemical cell will now be described by way of example of the improved method of manufacture.

Step 1: A sheet **101** of Melinex® (a chemically inert and electrically resistive Polyethylene Terephthalate ["PET"]) approximately 13 cm x 30 cm and 100 micron thick was laid flat on a sheet of release paper **102** and coated using a Number 2 MYAR bar to a thickness of 12 microns wet (approximately 2-5 microns dry) with a water-based heat activated adhesive **103** (ICI Novacoat system using catalyst:adhesive). The water was then evaporated by means of a hot air dryer leaving a contact adhesive surface. The sheet was then turned over on a release paper and the reverse side was similarly coated with the same adhesive **104**, dried, and a protective release paper **105** applied to the exposed adhesive-surface. The edges were trimmed to obtain a sheet uniformly coated on both sides with tacky contact adhesive protected by release paper.

Step 2: The sheet with protective release papers was cut into strips **107**, each about 18 mm x 210 mm (Figures 15-17).

Step 3: A strip **107** of adhesive-coated PET from Step 2 with release paper **102**, **105** on respective sides, was placed in a die assembly (not shown) and clamped. The die assembly was adapted to punch the strip with a locating hole **110** at each end and with for example 37 circular holes **111** each of 3.4 mm. diameter at 5 mm centers equi-spaced along a line between locating holes **110**. The area of each hole **111** is approximately 9 mm^2 (Figures 18 and 19).

Step 4: A sheet **112** of Mylar® PET approximately 21 cm square and 135 microns thick was placed in a sputter coating chamber for palladium coating **113**. The sputter coating took place under a vacuum of between 4 and 6 millibars and in an atmosphere of argon gas. Palladium was coated on the PET to a thickness of 100-1000 angstroms. There is thus formed a sheet **114** having a palladium sputter coating **113**.

Step 5: The palladium coated PET sheet **114** from Step 4 was then cut into strips **114** and **15** and a die was used to punch two location holes **16** in each strip, at one end (Figures 20, 21, and 22). Strips **114** and **115** differ only in dimension strips **114** being 25 mm x 210 mm and strips **115** being 23 mm x 210 mm.

Step 6: A spacer strip **107** prepared as in Step 3 was then placed in a jig (not shown) having two locating pins (one corresponding to each locating hole **110** of strip **107**) and the upper release paper **102** was removed. A strip **14** of palladium coated PET prepared as in Step **105** was then laid over the adhesive layer, palladium surface downwards, using the jig pins to align the locating holes **116** with the underlying PET-strip **107**. This combination was then passed

through a laminator comprising a set of pinch rollers; one of which was adapted to heat the side bearing a palladium coated PET strip **114**. The roller on the opposite side of the strip **107** was cooled. By this means, only the adhesive between the palladium of strip **114** and PET strip **107** was activated.

Step 7: PET strip **107** was then turned over and located in the jig with the release coating uppermost. The release coating was peeled off and second palladium coated strip **115** was placed palladium side down on the exposed adhesive surface using the locating pins to align the strips. This assembly was now passed again through the laminator of Step 6, this time with the hot roll adjacent the palladium coated Mylar® added in Step 7 so as to activate the intervening adhesive (Figures 23, 24, and 25).

Step 8: The assembly from Step 7 was returned to the die assembly and notches **116** punched in locations so as to extend between the circular holes **111** in the Melinex® PET and the strip edge **117**. Notches **116** extend so as to intercept the circumference of each circular cell. The strip was then guillotined to give individual "sensor strips", each strip being about 5 mm wide and each having one thin layer cavity cell (Figures 25, 27, and 28).

There is thus produced a cell as shown in Figures 26, 27, or 28. The cell comprises a first electrode consisting of PET layer **221**, a palladium layer **113**, an adhesive layer **103**, a PET sheet **101**. a second adhesive layer **104**, a second electrode comprising palladium layer **113**, and a PET layer **112**. Sheet **101** defines a cylindrical cell **111** having a thickness in the cell axial direction corresponding to the thickness of the Melinex® PET sheet layer **101** together with the thickness of adhesive layers **103** and **104**. The cell has circular palladium end walls. Access to the cell is provided at the side edge of the cell where notches **116** intersect cell **111**.

In preferred embodiments of the invention, a sample to be analysed is introduced to the cell by capillary action. The sample is placed on contact with notch **116** and is spontaneously drawn by capillary action into the cell, displaced air from the cell venting from the opposite notch **16**. A surfactant may be included in the capillary space to assist in drawing in the sample. The sensors are provided with connection means for example edge connectors whereby the sensors may be placed into a measuring circuit.

In a preferred embodiment this is achieved by making spacer **101** shorter than palladium supporting sheets **114**, **115** and by making one sheet **115** of shorter length than the other **114**. This forms a socket region **120** having contact areas **121**, **122** electrically connected with the

working and counter electrodes respectively. A simple tongue plug having corresponding engaging conduct surfaces can then be used for electrical connection. Connectors of other form may be devised.

Chemicals for use in the cell may be supported on the cell electrodes or walls, may be supported on an independent support contained within the cell or may be self-supporting. In one embodiment, chemicals for use in the cell are printed onto the palladium surface of the electrode immediately after Step 1 at which stage the freshly-deposited palladium is more hydrophilic. For example, a solution containing 0.2 molar potassium ferricyanide and 1% by weight of glucose oxidase dehydrogenase may be printed on to the palladium surface. Desirably, the chemicals are printed only in the areas which will form a wall of the cell and for preference the chemicals are printed on the surface by means of an ink jet printer. In this manner, the deposition of chemicals may be precisely controlled.

If desired, chemicals which are desirably separated until required for use may be printed respectively on the first and second electrodes. For example, a GOD/ferrocyanide composition can be printed on one electrode and a buffer on the other. Although it is highly preferred to apply the chemicals to the electrodes prior to assembly into a cell, chemicals may also be introduced into the cell as a solution after Step 6 or Step 8 by pipette in the traditional manner and the solvent subsequently is removed by evaporation or drying.

Chemicals need not be printed on the cell wall or the electrodes and may instead be impregnated into a gauze, membrane, non-woven fabric or the like contained within, or filling, the cavity (e.g. inserted in cell **111** prior to Steps 6 or 7). In another embodiment the chemicals are formed into a porous mass which may be introduced into the cell as a pellet or granules. Alternatively, the chemicals may be introduced as a gel.

In a second embodiment of the invention a laminate **121** is first made from a strip **114** as obtained in Step 5 adhesively sandwiched between two strips **107** as obtained from Step 3. Laminate **120** is substituted for sheet **101** in Step 5 and assembled with electrodes as in Steps 6 and 7. There is thus obtained a cell as shown in Figure 29 which differs from that of Figures 23 to 25 in that the cell has an annular electrode disposed between the first and second electrode. This electrode can for example be used as a reference electrode. It will be understood that in mass production of the cell, the parts may be assembled as a laminate on a continuous line. For example, a continuous sheet **101** of PET could be first punched and then adhesive could be

applied continuously by printing on the remaining sheet. Electrodes (pre-printed with chemical solution and dried) could be fed directly as a laminate onto the adhesive coated side. Adhesive could then be applied to the other side of the punched core sheet and then the electrode could be fed as a laminate onto the second side.

The adhesive could be applied as a hot melt interleaving film. Alternatively, the core sheet could first be adhesive coated and then punched. By drying chemicals on each electrode prior to the gluing step the electrode surface is protected from contamination. Although the cell has been described with reference to Mylar® and Melinex® PET, other chemically inert and electrically resistive materials may be utilised and other dimensions chosen. The materials used for spacer sheet **101** and for supporting the reference and counter electrodes may be the same or may differ one from the other. Although the invention has been described with reference to palladium electrodes, other metals such as platinum, silver, gold, copper or the like may be used and silver may be reacted with a chloride to form a silver/silver chloride electrode or with other halides. The electrodes need not be of the same metal.

Although the use of heat activated adhesives has been described, the parts may be assembled by use of hot melt adhesives, fusible laminates and other methods.

The dimensions of the sensor may readily be varied according to requirements. While it is greatly preferred that the electrodes cover the cell end openings, in other embodiments (not illustrated) the electrodes do not entirely cover the cell end openings. In that case it is desirable that the electrodes be in substantial overlying registration. Preferred forms of the invention in which the electrodes cover the apertures of cell **111** have the advantages that the electrode area is precisely defined simply by punching hole **111**. Furthermore the electrodes so provided are parallel, overlying, of substantially the same area, and are substantially or entirely devoid of "edge" effects.

Although in the embodiments described each sensor has one cell cavity, sensors may be provided with two or more cavities. For example, a second cavity may be provided with a predetermined quantity of the analyte and may function as a reference cell. As will be apparent to those skilled in the art from the teaching herein contained, a feature of one embodiment herein described may be combined with features of other embodiments herein described. Although the sensor has been described with reference to palladium electrodes and a GOD/ferrocyanide

chemistry, it will be apparent to those skilled in the art that other chemistries, and other materials of construction may be employed without departing from the principles herein taught.

The electrochemical means for measuring the ferrocyanide concentration after complete reaction can be considered by reference to Figure 4.

In a thin layer cell the initial concentration of ferrocyanide and ferricyanide (after 'enzymatic' reaction is complete) is equal throughout the cell (the axis of interest being that between the electrodes). The concentration profile of ferrocyanide is given in Figure 5.

When a particular potential is applied across the cell ferricyanide is converted to ferrocyanide at the cathode and ferrocyanide is converted to ferricyanide at the anode.

The chemistry is so arranged that after complete reaction there is still an excess of ferricyanide compared to ferrocyanide. For this reason the process that limits the complete electrochemical process is the conversion of ferrocyanide to ferricyanide at the anode, simply because ferrocyanide is at a significantly lower concentration. Further the rate limiting step for the reaction of ferrocyanide is the diffusion of ferrocyanide to the anode. After a period of time a steady-state is achieved, wherein the concentration profile of ferrocyanide and ferricyanide remains constant (see Figure 5).

Therefore there are two limiting situations: initially **20** the ferrocyanide is evenly distributed throughout the cell. Then after a known potential is applied across the cell for a period of time a steady-state concentration profile of ferrocyanide is achieved. The 'transient' **22** reflects the measured cur-rent across the cell as the concentration adjusts from the initial situation to the final steady state situation **23**. This is shown as a function of time in Figure 6. It has been found that the change in the current with time during this 'transient' period is dependent upon the total concentration of ferrocyanide and the diffusion coefficient of ferrocyanide.

By solving the diffusion equations for this situation, it can be shown that the transient can be adequately described by the following equation over a restricted calculable time range.

$$\ln\left(\frac{i}{i_s} - 1\right) = \frac{-4\pi^2 Dt}{L^2} + \ln(2) \qquad \text{Eqn 1}$$

where i is the measured current, i_s is the current at steady-state, D the diffusion coefficient of ferrocyanide in the cell, L the separation distance between the anode and cathode, and t is time.

This is a simple solution of the general diffusion equation. However, it may be possible to use other solutions.

The final current at steady state also depends upon the total concentration of ferrocyanide and the diffusion coefficient of ferrocyanide. The steady state current can also be modeled by diffusion theory and is given by:

$$i_{ss} = \frac{2DFCA}{L} \qquad \text{Eqn 2}$$

where F is the Faraday constant, C the initial concentration of ferrocyanide, and A the area of the working electrode. By initial concentration is meant the unperturbed concentration (shown as **20** in Figure 5).

Analysis of the current observed during the transient and also at steady state allows calculation of both the concentration and diffusion coefficient of ferrocyanide, and thus also the initial glucose concentration.

This analysis is achieved by plotting:

$$\ln\left(\frac{i}{i_s} - l\right) = \qquad \text{Eqn 3}$$

versus time which is substantially linear over a restricted and calculable time range and thus can be analysed for example by linear least squares regression. Since L is a constant for a given cell, measurement of i as a function of time and of i_{ss} thus enables the value of the diffusion coefficient of the redox mediator to be calculated and the concentration of the analyte to be determined.

Another possible way to analyse the data is to use the variation of current with time soon after the potential step is applied to the electrodes. In this time period the current can be adequately described by the Cottrell equation. That is:

$$i - FAD^{1/2}C/(pi^{1/2}t^{1/2}) \qquad \text{Eqn 4}$$

By least squares regression on a plot of i versus $1/t^{1/2}$ a value of $FAD1^{/2}C/pi^{1/2}$ can be estimated from the slope of this plot. The steady state current i_{ss} is given as before, so by combining the

slope of the plot given above with the steady state current a value of the concentration of the ferrocyanide, independent of the diffusion coefficient of the ferrocyanide in the cell can be estimated. This is given by:

$$C = 2\text{slope}^2 pi/(FALi_{ss}) \qquad \text{Eqn 5}$$

In an example according to the preferred embodiments, a sample of blood is admitted to a thin layer cell containing a GOD/ferrocyanide system such as previously described with reference to Figures 12, 13, and 14. As illustrated in Figure 6, after allowing a short time **20** for reaction, an electric potential is applied between the electrodes, current flow commences when the potential is applied **21** but then falls as a transient **22** towards a steady state level **23**. The diffusion coefficient and/or glucose concentration are derived by measuring current as a function of time and by estimating the steady state current.

According to the preferred embodiments, the current is then interrupted, or reversed in polarity, for example by means of a suitable switch. If the polarity is reversed, a second transient is then observed, and a second steady state is reached after a further period of time although the profile is reversed. The underlying change in ferrocyanide concentration profile across the cell is shown schematically in Figure 7. The initial concentration profile prior to current reversal is **23**. The new steady state concentration profile is shown at **25**. The transient concentration profiles are exemplified at **24**.

By solving the diffusion equations for this situation, it can be shown that the transient current is described by.

$$\ln\left(\frac{i}{i_s} - 1\right) = \frac{-4\pi^2 Dt}{L^2} + \ln(4) \qquad \text{Eqn 6}$$

It is therefore simple to re-estimate the diffusion coefficient and concentration under the reversed polarity conditions. In theory the results should be independent of the type of transient or polarity. In practice, the results may differ due to factors affecting the transient such as sample inhomogeneity, state of the electrodes, or more importantly, due to asymmetries in the cell construction. This measure is therefore useful for cell diagnosis and also enables greater accuracy by allowing repetitive measurement and averaging with reverse polarities.

Similarly, if the potential is interrupted after steady state is reached, the initial concentration profile will be re-established in a short time (for example 4 seconds).
Once the initial state is re-established (or approximated) the potential can be re-applied and the procedure repeated without current reversal. Figure 9 shows a plot of current versus time similar to that of Figure 6 but having the potential interrupted at **26** and reapplied after 15 seconds at **27** yielding a new transient current **28** and then a state **29**.

As stated previously, the presence of oxygen in the blood is an interference since the concentration of final ferrocyanide is then not directly proportional to the initial glucose. Instead the initial glucose is related both to the final concentration of ferrocyanide plus hydrogen peroxide. However, it was found that hydrogen peroxide can be oxidised at the anode at a known potential which is higher than that for the ferrocyanide/ferricyanide redox reaction. The total electrochemical path is given in Figure 10. The hydrogen peroxide reaction is:

hydrogen peroxide $\rightarrow$ oxygen + 2 H^+ + 2 e^- reaction 7

If, during the period of enzyme reaction a potential is applied (Figure 11) across the cell that is sufficient to oxidise hydrogen peroxide, then the following will happen during that period:
(a) glucose will be reacted to gluconic acid.
(b) ferrocyanide and hydrogen peroxide will result.
(c) the ferrocyanide/ferricyanide redox will eventually reach steady state.
(d) the peroxide will be oxidised at the anode and the electrons used to convert ferricyanide to ferrocyanide.

In total, after a period of time (approximately 2½ seconds in Figure 11) at a constant potential all the peroxide will be converted to oxygen (which is then a catalyst, and will return to complete more enzyme chemistry until glucose is exhausted), and the electrons utilised to convert ferricyanide to ferrocyanide.

At this stage (60 seconds in Figure 11) a reverse transient is applied. That is, the polarity of the cells is switched. but now at the lower potential suitable for the ferricyanide/ferrocyanide redox reaction. The final steady state ferrocyanide will once again reflect the initial glucose concentration. This can be analysed in the previously described manner to determine the total concentration of glucose in the initial sample.

Using the method of the preferred embodiments, the reaction phase of the test can be monitored in situ electrochemically without interfering with the measurement phase.

When the reaction is complete one can proceed to measurement without further delay.

The wait time will vary from test to test and will be the minimum necessary for any particular sample and cell, taking account of changes in enzyme activity from cell to cell as well as different temperatures and glucose concentrations. This is in stark contrast to prior art in which measurement is delayed until the maximum time required for reaction after allowing for all these factors.

In the present method the reaction phase is monitored by applying a potential between the two electrodes of, for example, -300 mV as soon as the cell begins to fill with sample.

A linear concentration profile of the reduced mediator is soon achieved across the cell. As more reduced mediator is produced by the enzyme reaction with glucose this linear concentration profile becomes steeper and the current increases. When the reaction is complete the current no longer increases. This point can be detected by well known electronic means and the measurement phase of the test can then be commenced.

The end-point of the reaction can also be estimated by fitting a theoretical kinetic equation to the current versus time curve generated during this part of the test.

This equation can predict the degree of completion of the reaction at any time. so would allow knowledge of when the end-point would occur without having to wait to get there.

This would further shorten the test time. For example, one could fit an equation to the measured prepulse current versus time curve. This equation could then predict that at time X the reaction will be, for example, 90% complete. If one measures the concentration at time X one would then divide the answer by 0.90 to get the true concentration.

The measurement of concentration in this system is done by reversing the potential, i.e. applying +300 mV between the electrodes. A current versus time curve will then occur, which is the same as that of the second transient in a double transient experiment le by transforming the current 1 measured during the measurement phase one can obtain a plot of $\ln(i/i_{ss} - 1)$ versus time which will have a slope of $-4pi^2D/l^2$ and an intercept ln(4). The normal analysis can then be used to obtain the concentration of glucose.

In some situations it may be difficult or impossible to know the distance between the electrodes in the electrochemical cell. For example, very small separations (ca. 10 microns) may

be very difficult to manufacture or measure reproducibly. In these situations the use of information from two adjoining cells can be used to calculate the concentration of an analyte in a sample without knowledge of the cell separation if one of the cells contains a known concentration of the analyte or the corresponding reduced mediator prior to sample addition. Alternatively, a known quantity of this analyte or reduced mediator can be added to the sample destined for one of the two cells prior to addition of the sample to the cell. Another variation is if both cells contain a pre-determined analyte or reduced mediator concentration but each has a different concentration. Yet another variation is if two different predetermined quantities of the analyte or reduced mediator are added to two aliquots of the sample, which are then added to the adjoining cells.

The two electrochemical cells are then used in the normal fashion, and from each cell the following quantities are measured: steady state current (i_{ss}) and the slope of the straight line defined by $\ln(i/i_{ss} - 1)$ versus time, where i is the measured current. With a knowledge of these values and also a knowledge of the difference in concentration of the analyte or reduced mediator between the two cells, which is known (it is equal to that value purposely added to one cell), it is possible to calculate the concentration of analyte or reduced mediator in the sample, without any knowledge of the separation distance of the electrodes.

The above can be used in conjunction with a third cell that is used to measure the background current or concentration due to current caused by, for example, reduced mediator formed by the application and drying of the chemistry, catalytic effect of the metal surface, oxidation of the metal surface, sample components that have effects on the analyte or mediator, electrochemically responsive components of the sample etc. This background concentration or current would be subtracted from the values measured from the two cells discussed above to calculate the true values for each cell resulting from the analyte in the sample, and in one case also the analyte or reduced mediator purposely added to the cell or the sample.

As will be apparent to those skilled in the art, from the teaching hereof the method is suitable for use with automatic measuring apparatus. Cells of the kind described may be provided with electrical connectors to an apparatus provide with a microprocessor or other programmed electronic control and display circuits which are adapted to make the required measurements perform the required calculations and to display the result. The method may be used to measure the concentration of analytes other than glucose and in liquids other than blood.

The method may be conducted using cells of other design and/or construction and using known catalysts and redox systems other than that exemplified.--

Appl. No. : **Unknown**
Filed : **Herewith**

REMARKS

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE." The specification has been amended to incorporate selected text of International Patent Application Nos. PCT/AU/00723 and PCT/AU/00723, the contents of which were incorporated by reference in the parent application. A declaration by the practitioner representing the Applicants stating that the amendatory material consists of the same material incorporated by reference in the present application is enclosed. No new matter has been added.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

Deleted text is indicated by **[bracketed boldface]**. Added text is indicated by **underlined boldface**.

IN THE SPECIFICATION:

The paragraph beginning at page 1, line 3 has been amended as follows:

This application is a continuation **of Application No. 09/659,470, filed on 11 September 11 2000, which is a continuation, under 35 U.S.C. § 120,** of **[copending PCT] International Patent** Application No. PCT/AU99/00152, filed on 11 March 1999, **under the Patent Cooperation Treaty (PCT), which was published by the International Bureau in English on 16 September 1999, which designates the U.S. and claims the benefit of [which claimed priority from]** Australian Patent Application No. PP2388, filed on 12 March 1998.

The following paragraphs have been inserted at page 4, after line 3:

--Figure 4 exemplifies the reactions taking place in a cell according to preferred embodiments.

Figure 5 illustrates the concentration profiles across an electrochemical cell according to the preferred embodiments before the application of an electrical potential, after application of the potential and prior to reaching steady state, and at steady state.

Figure 6 shows the time dependence of current prior to and after application of electrical potential.

Figure 7 shows the ferrocyanide concentration profiles across an electrochemical cell according to the preferred embodiments prior to a polarity reversal, after reversal and prior to reaching a steady state, and at steady state.

Figure 8 shows the time dependence of current prior to and after a polarity reversal.

Figure 9 shows the time dependence of current prior to and after an interruption of applied potential of 15 seconds.

Figure 10 shows the reactions in an electrochemical cell with peroxide oxidation.

Figure 11 shows the time dependence of current when an initial potential sufficient to oxidise hydrogen peroxide is applied.

Figure 12 describes the cell of Figure 10, which is suitable for use in the method of the preferred embodiments, in plan view (not to scale).

Figure 13 describes an embodiment of a cell suitable for use in the preferred embodiments in cross-section view on line 10-10 of Figure 12 (not to scale).

Figure 14 describes the cell of Figure 10, which is suitable for use in the method of the preferred embodiments, in end section view (not to scale).

Figure 15 show the product of manufacturing Step 2 in plan.

Figure 16 shows the product of Figure 15 in side elevation.

Figure 17 shows the product of Figure 15 in end elevation.

Figure 18 shows the product of manufacturing Step 3 in plan.

Figure 19 shows the product of Figure 18 in cross-section on line 105-105 of Figure 18.

Figure 20 shows the product of manufacturing Step 5 in plan.

Figure 21 shows the product of Figure 20 in side elevation.

Figure 22 shows the product of Figure 20 in end elevation.

Figure 23 shows the product of manufacturing Step 7 in plan.

Figure 24 is a cross-section of Figure 23 on line 110-110.

Figure 25 shows the product of Figure 23 in end elevation.

Figure 26 shows a cell according to the invention in plan.

Figure 27 shows the call of Figure 26 in side elevation.

Figure 28 shows the cell of Figure 26 in end elevation.

Figure 29 shows a scrap portion of a second embodiment of the invention in enlarged section.--

The paragraphs beginning at page 8, line 3 has been amended as follows:

When using cells such as described in our co-pending applications **(see, e.g., Figures 12, 13, and 14),** the sample volumes are very small and heating can be achieved with low energy input.

The following paragraphs have been inserted at page 8, after line 4:

--It is known to measure the concentration of a component to be analysed in an aqueous liquid sample by placing the sample into a reaction zone in an electrochemical cell comprising two electrodes having an impedance which renders them suitable for amperometric measurement. The component to be analysed is allowed to react directly with an electrode, or directly or indirectly with a redox reagent whereby to form an oxidisable (or reducible) substance in an amount corresponding to the concentration of the component to be analysed. The quantity of the oxidisable (or reducible) substance present is then estimated electrochemically. Generally this method requires sufficient separation of the electrodes so that electrolysis products at one electrode cannot reach the other electrode and interfere with the processes at the other electrode during the period of measurement.

In PCT/AU96/00365 is described a novel method for determining the concentration of the reduced (or oxidised) form of a redox species in an electrochemical cell of the kind comprising a working electrode and a counter (or counter/reference) electrode spaced from the working electrode. The method involves applying an electrical potential difference between the electrodes, spacing the working electrode from the counter electrode so that reaction products from the counter electrode arrive at the working electrode and selecting the potential of the working electrode so that the rate of electro-oxidation of the reduced form of the species (or of electro-reduction of the oxidised form) is diffusion controlled. By determining the current as a function of time after application of the potential and prior to achievement of a steady state current and then estimating the magnitude of the steady state current, the method previously described allows the diffusion coefficient and/or the concentration of the reduced (oxidised) form of the species to be estimated.

PCT/AU96/00365 exemplifies this method with reference to use of a "thin layer" cell employing a GOD/Ferrocyanide system. As herein used, the term "thin layer electrochemical

cell" refers to a cell having closely spaced electrodes such that reaction products from the counter electrode arrive at the working electrode, in practice, the separation of electrodes in such a cell for measuring glucose in blood will be less than 500 microns, and preferably less than 200 microns.

The chemistry used in the exemplified electrochemical cell is as follows:

glucose + GOD → gluconic acid + GOD* reaction 1

GOD* + 2 ferricyanide → GOD + 2 ferrocyanide reaction 2

where GOD is the enzyme glucose oxidase and GOD* is the 'activated' enzyme. Ferricyanide ($[Fe(CN)_6]^{3-}$) is the 'mediator' which returns the GOD* to its catalytic state. GOD, an enzyme catalyst, is not consumed during the reaction so long as excess mediator is present. Ferrocyanide ($[Fe(CN)_6]^{4-}$) is the product of the total reaction.

Ideally there is initially no ferrocyanide, although in practice there is often a small quantity. After reaction is complete the concentration of ferrocyanide (measured electrochemically) indicates the initial concentration of glucose. The total reaction is the sum of reactions 1 and 2:

GOD glucose + 2 ferricyanide → gluconic acid + 2 ferrocyanide reaction 3

"Glucose" refers specifically to β-D-glucose.

The prior art suffers from a number of disadvantages. Firstly, sample size required is greater than desirable. It would be generally preferable to be able to make measurements on samples of reduced volume since this in turn enables use of less invasive methods to obtain samples.

Secondly, it would be generally desirable to the accuracy of measurement and to eliminate or reduce variations due, for example, to cell asymmetry or other factors introduced during mass production of microcells.

Thirdly, it would be generally desirable to reduce the time that is required in which to obtain a measurement. The test protocols used in current commercially available electrochemical glucose sensors involve a predetermined wait period at the beginning of the test during which the

enzyme reacts with the glucose to produce the specie that is sensed electrochemically. This initial period is fixed at the maximum necessary to achieve the desired reaction under all conditions of use.

Fourthly, it would be desirable to eliminate variations due to oxygen. Oxygen can be plentiful in blood, either dissolved in the plasma, or bound in hemoglobin. It can also be introduced during "finger sticking," where a blood drop of small volume and high surface area is exposed to the atmosphere prior to introduction to a cell. Oxygen can interfere since oxygen is a mediator for GOD. The reaction is as follows:

glucose + GOD → gluconic acid + GOD* reaction 4

GOD* + oxygen + water → GOD + hydrogen peroxide reaction 5

The total reaction is given by:

$$\text{glucose} + \text{water} + \text{oxygen} \xrightarrow{\text{GOD}} \text{gluconic acid} + \text{hydrogen peroxide} \quad \text{reaction 6}$$

In most situations the complication of oxygen also acting as a mediator is unwanted simply because the concentration of final ferrocyanide no longer is directly proportional to the concentration of initial glucose. Instead, the initial glucose concentration is then related to both the final concentration of ferrocyanide and of hydrogen peroxide.

A method is provided for determining the concentration of a reduced (or oxidised) form of a redox species in an electrochemical cell of the kind comprising a working electrode and a counter electrode spaced from the working electrode by a predetermined distance, said method comprising the steps of:

(a) applying an electric potential between the electrodes, wherein the electrodes are spaced so that reaction products from the counter electrode arrive at the such that the rate of the electro-oxidation of the reduced form (or oxidised form) of the redox species is diffusion controlled,

(b) determining current as a function of time after application of the potential and prior to achievement of a steady state current,

(c) estimating the magnitude of the steady state current,

(d) interrupting, or reversing the polarity, of the potential,

(e) repeating step (b) and step (c).

It was discovered that if the polarity is reversed (i.e., the anode becomes the cathode and vice versa) after the initial steady state current is achieved, then a second transient current can be observed and after a period of time a second steady state is achieved. This has proved useful for diagnosing, and for reducing the effects of, cell asymmetry and other factors which influence the transient current. It also permits greater reliability and/or accuracy of estimation by allowing measurements to be made repetitively using reverse polarities. Likewise, if the potential is interrupted for a time sufficient for the concentration profile to relax to a random state and is then reapplied, steps (b) and (c) can be repeated.

A method is provided for measuring the concentration of glucose in a sample by means of a cell having a working electrode, a counter electrode, an enzyme catalyst, and a redox mediator, comprising the steps of operating the cell at a potential higher than that of the redox reaction so as to oxidise hydrogen peroxide at the anode and then conducting a method as described above.

By this means the interference of oxygen can be ameliorated as explained in more detail hereinafter.

A method is provided wherein the sample is allowed to react with an enzyme catalyst and a redox mediator comprising the steps of:

(a) applying a potential between the electrodes before or during filling of the cell,

(b) measuring the increase in current as a function of time,

(c) determining or predicting from the measurement in step (b) the time of completion of reaction with said catalyst, and

(d) then interrupting or reversing the polarity of the potential.

An electrochemical cell suitable for use in preferred embodiments is depicted schematically (not to scale) in Figures 12, 13, and 14. The cell comprises a polyester core **4** approximately 18 mm x 5 mm and 100 micron thick and having a circular aperture **8** of 3.4 mm diameter. Aperture **8** defines a cylindrical cell side wall **10**. Adhered to one side of core **4** is a polyester sheet **1** having a sputter coating of palladium **2**. The sputter coating took place at between 4 and 6 millibar pressure in an atmosphere of argon gas to give a uniform coating thickness of 100-1000 angstroms. The sheet is adhered by means of an adhesive **3** to core **4** with palladium **2** adjacent core **4** and covering aperture **8**.

A second polyester sheet **7** having a second sputter coating of palladium **6** is adhered by means of contact adhesive **5** to the other side of core **4** and covering aperture **8**. There is thereby defined a cell having cylindrical side wall **10** and closed each end by palladium metal. The assembly is notched at **9** to provide for a solution to be admitted to the cell or to be drawn in by wicking or capillary action and to allow air to escape.

The metal films **2, 6** are connected with suitable electrical connections or formations whereby potentials may be applied and currently measured. The cell is furnished with GOD and ferrocyanide in dry form. In use according to the method a drop of blood is drawn into the cell at **9** by capillary action and allowed to react.

The construction of a thin layer electrochemical cell will now be described by way of example of the improved method of manufacture.

Step 1: A sheet **101** of Melinex® (a chemically inert and electrically resistive Polyethylene Terephthalate ["PET"]) approximately 13 cm x 30 cm and 100 micron thick was laid flat on a sheet of release paper **102** and coated using a Number 2 MYAR bar to a thickness of 12 microns wet (approximately 2-5 microns dry) with a water-based heat activated adhesive **103** (ICI Novacoat system using catalyst:adhesive). The water was then evaporated by means of a hot air dryer leaving a contact adhesive surface. The sheet was then turned over on a release paper and the reverse side was similarly coated with the same adhesive **104**, dried, and a protective release paper **105** applied to the exposed adhesive-surface. The edges were trimmed to obtain a sheet uniformly coated on both sides with tacky contact adhesive protected by release paper.

Step 2: The sheet with protective release papers was cut into strips **107**, each about 18 mm x 210 mm (Figures 15-17).

Step 3: A strip **107** of adhesive-coated PET from Step 2 with release paper **102, 105** on respective sides, was placed in a die assembly (not shown) and clamped. The die assembly was adapted to punch the strip with a locating hole **110** at each end and with for example 37 circular holes **111** each of 3.4 mm. diameter at 5 mm centers equi-spaced along a line between locating holes **110**. The area of each hole **111** is approximately 9 mm^2 (Figures 18 and 19).

Step 4: A sheet **112** of Mylar® PET approximately 21 cm square and 135 microns thick was placed in a sputter coating chamber for palladium coating **113**. The sputter coating took place under a vacuum of between 4 and 6 millibars and in an atmosphere of argon gas.

Palladium was coated on the PET to a thickness of 100-1000 angstroms. There is thus formed a sheet **114** having a palladium sputter coating **113**.

Step 5: The palladium coated PET sheet **114** from Step 4 was then cut into strips **114** and **15** and a die was used to punch two location holes **16** in each strip, at one end (Figures 20, 21, and 22). Strips **114** and **115** differ only in dimension strips **114** being 25 mm x 210 mm and strips **115** being 23 mm x 210 mm.

Step 6: A spacer strip **107** prepared as in Step 3 was then placed in a jig (not shown) having two locating pins (one corresponding to each locating hole **110** of strip **107**) and the upper release paper **102** was removed. A strip **14** of palladium coated PET prepared as in Step **105** was then laid over the adhesive layer, palladium surface downwards, using the jig pins to align the locating holes **116** with the underlying PET-strip **107**. This combination was then passed through a laminator comprising a set of pinch rollers; one of which was adapted to heat the side bearing a palladium coated PET strip **114**. The roller on the opposite side of the strip **107** was cooled. By this means, only the adhesive between the palladium of strip **114** and PET strip **107** was activated.

Step 7: PET strip **107** was then turned over and located in the jig with the release coating uppermost. The release coating was peeled off and second palladium coated strip **115** was placed palladium side down on the exposed adhesive surface using the locating pins to align the strips. This assembly was now passed again through the laminator of Step 6, this time with the hot roll adjacent the palladium coated Mylar® added in Step 7 so as to activate the intervening adhesive (Figures 23, 24, and 25).

Step 8: The assembly from Step 7 was returned to the die assembly and notches **116** punched in locations so as to extend between the circular holes **111** in the Melinex® PET and the strip edge **117**. Notches **116** extend so as to intercept the circumference of each circular cell. The strip was then guillotined to give individual "sensor strips", each strip being about 5 mm wide and each having one thin layer cavity cell (Figures 25, 27, and 28).

There is thus produced a cell as shown in Figures 26, 27, or 28. The cell comprises a first electrode consisting of PET layer **221**, a palladium layer **113**, an adhesive layer **103**, a PET sheet **101**. a second adhesive layer **104**, a second electrode comprising palladium layer **113**, and a PET layer **112**. Sheet **101** defines a cylindrical cell **111** having a thickness in the cell axial direction corresponding to the thickness of the Melinex® PET sheet layer **101** together with the thickness

of adhesive layers **103** and **104**. The cell has circular palladium end walls. Access to the cell is provided at the side edge of the cell where notches **116** intersect cell **111**.

In preferred embodiments of the invention, a sample to be analysed is introduced to the cell by capillary action. The sample is placed on contact with notch **116** and is spontaneously drawn by capillary action into the cell, displaced air from the cell venting from the opposite notch **16**. A surfactant may be included in the capillary space to assist in drawing in the sample. The sensors are provided with connection means for example edge connectors whereby the sensors may be placed into a measuring circuit.

In a preferred embodiment this is achieved by making spacer **101** shorter than palladium supporting sheets **114, 115** and by making one sheet **115** of shorter length than the other **114**. This forms a socket region **120** having contact areas **121, 122** electrically connected with the working and counter electrodes respectively. A simple tongue plug having corresponding engaging conduct surfaces can then be used for electrical connection. Connectors of other form may be devised.

Chemicals for use in the cell may be supported on the cell electrodes or walls, may be supported on an independent support contained within the cell or may be self-supporting. In one embodiment, chemicals for use in the cell are printed onto the palladium surface of the electrode immediately after Step 1 at which stage the freshly-deposited palladium is more hydrophilic. For example, a solution containing 0.2 molar potassium ferricyanide and 1% by weight of glucose oxidase dehydrogenase may be printed on to the palladium surface. Desirably, the chemicals are printed only in the areas which will form a wall of the cell and for preference the chemicals are printed on the surface by means of an ink jet printer. In this manner, the deposition of chemicals may be precisely controlled.

If desired, chemicals which are desirably separated until required for use may be printed respectively on the first and second electrodes. For example, a GOD/ferrocyanide composition can be printed on one electrode and a buffer on the other. Although it is highly preferred to apply the chemicals to the electrodes prior to assembly into a cell, chemicals may also be introduced into the cell as a solution after Step 6 or Step 8 by pipette in the traditional manner and the solvent subsequently is removed by evaporation or drying.

Chemicals need not be printed on the cell wall or the electrodes and may instead be impregnated into a gauze, membrane, non-woven fabric or the like contained within, or filling,

the cavity (e.g. inserted in cell **111** prior to Steps 6 or 7). In another embodiment the chemicals are formed into a porous mass which may be introduced into the cell as a pellet or granules. Alternatively, the chemicals may be introduced as a gel.

In a second embodiment of the invention a laminate **121** is first made from a strip **114** as obtained in Step 5 adhesively sandwiched between two strips **107** as obtained from Step 3. Laminate **120** is substituted for sheet **101** in Step 5 and assembled with electrodes as in Steps 6 and 7. There is thus obtained a cell as shown in Figure 29 which differs from that of Figures 23 to 25 in that the cell has an annular electrode disposed between the first and second electrode. This electrode can for example be used as a reference electrode. It will be understood that in mass production of the cell, the parts may be assembled as a laminate on a continuous line. For example, a continuous sheet **101** of PET could be first punched and then adhesive could be applied continuously by printing on the remaining sheet. Electrodes (pre-printed with chemical solution and dried) could be fed directly as a laminate onto the adhesive coated side. Adhesive could then be applied to the other side of the punched core sheet and then the electrode could be fed as a laminate onto the second side.

The adhesive could be applied as a hot melt interleaving film. Alternatively, the core sheet could first be adhesive coated and then punched. By drying chemicals on each electrode prior to the gluing step the electrode surface is protected from contamination. Although the cell has been described with reference to Mylar® and Melinex® PET, other chemically inert and electrically resistive materials may be utilised and other dimensions chosen. The materials used for spacer sheet **101** and for supporting the reference and counter electrodes may be the same or may differ one from the other. Although the invention has been described with reference to palladium electrodes, other metals such as platinum, silver, gold, copper or the like may be used and silver may be reacted with a chloride to form a silver/silver chloride electrode or with other halides. The electrodes need not be of the same metal.

Although the use of heat activated adhesives has been described, the parts may be assembled by use of hot melt adhesives, fusible laminates and other methods.

The dimensions of the sensor may readily be varied according to requirements. While it is greatly preferred that the electrodes cover the cell end openings, in other embodiments (not illustrated) the electrodes do not entirely cover the cell end openings. In that case it is desirable that the electrodes be in substantial overlying registration. Preferred forms of the invention in

which the electrodes cover the apertures of cell **111** have the advantages that the electrode area is precisely defined simply by punching hole **111**. Furthermore the electrodes so provided are parallel, overlying, of substantially the same area, and are substantially or entirely devoid of "edge" effects.

Although in the embodiments described each sensor has one cell cavity, sensors may be provided with two or more cavities. For example, a second cavity may be provided with a predetermined quantity of the analyte and may function as a reference cell. As will be apparent to those skilled in the art from the teaching herein contained, a feature of one embodiment herein described may be combined with features of other embodiments herein described. Although the sensor has been described with reference to palladium electrodes and a GOD/ferrocyanide chemistry, it will be apparent to those skilled in the art that other chemistries, and other materials of construction may be employed without departing from the principles herein taught.

The electrochemical means for measuring the ferrocyanide concentration after complete reaction can be considered by reference to Figure 4.

In a thin layer cell the initial concentration of ferrocyanide and ferricyanide (after 'enzymatic' reaction is complete) is equal throughout the cell (the axis of interest being that between the electrodes). The concentration profile of ferrocyanide is given in Figure 5.

When a particular potential is applied across the cell ferricyanide is converted to ferrocyanide at the cathode and ferrocyanide is converted to ferricyanide at the anode.

The chemistry is so arranged that after complete reaction there is still an excess of ferricyanide compared to ferrocyanide. For this reason the process that limits the complete electrochemical process is the conversion of ferrocyanide to ferricyanide at the anode, simply because ferrocyanide is at a significantly lower concentration. Further the rate limiting step for the reaction of ferrocyanide is the diffusion of ferrocyanide to the anode. After a period of time a steady-state is achieved, wherein the concentration profile of ferrocyanide and ferricyanide remains constant (see Figure 5).

Therefore there are two limiting situations: initially **20** the ferrocyanide is evenly distributed throughout the cell. Then after a known potential is applied across the cell for a period of time a steady-state concentration profile of ferrocyanide is achieved. The 'transient' **22** reflects the measured cur-rent across the cell as the concentration adjusts from the initial situation to the final steady state situation **23**. This is shown as a function of time in Figure 6. It has been

found that the change in the current with time during this 'transient' period is dependent upon the total concentration of ferrocyanide and the diffusion coefficient of ferrocyanide.

By solving the diffusion equations for this situation, it can be shown that the transient can be adequately described by the following equation over a restricted calculable time range.

$$\ln\left(\frac{i}{i_s} - 1\right) = \frac{-4\pi^2 Dt}{L^2} + \ln(2) \qquad \text{Eqn 1}$$

where i is the measured current, i_s is the current at steady-state, D the diffusion coefficient of ferrocyanide in the cell, L the separation distance between the anode and cathode, and t is time.

This is a simple solution of the general diffusion equation. However, it may be possible to use other solutions.

The final current at steady state also depends upon the total concentration of ferrocyanide and the diffusion coefficient of ferrocyanide. The steady state current can also be modeled by diffusion theory and is given by:

$$i_{ss} = \frac{2DFCA}{L} \qquad \text{Eqn 2}$$

where F is the Faraday constant, C the initial concentration of ferrocyanide, and A the area of the working electrode. By initial concentration is meant the unperturbed concentration (shown as **20** in Figure 5).

Analysis of the current observed during the transient and also at steady state allows calculation of both the concentration and diffusion coefficient of ferrocyanide, and thus also the initial glucose concentration.

This analysis is achieved by plotting:

$$\ln\left(\frac{i}{i_s} - 1\right) = \qquad \text{Eqn 3}$$

versus time which is substantially linear over a restricted and calculable time range and thus can be analysed for example by linear least squares regression. Since L is a constant for a given cell, measurement of i as a function of time and of i_{ss} thus enables the value of the diffusion

coefficient of the redox mediator to be calculated and the concentration of the analyte to be determined.

Another possible way to analyse the data is to use the variation of current with time soon after the potential step is applied to the electrodes. In this time period the current can be adequately described by the Cottrell equation. That is:

$i - FAD^{1/2}C/(pi^{1/2}t^{1/2})$ Eqn 4

By least squares regression on a plot of i versus $1/t^{1/2}$ a value of $FAD1^{/2}C/pi^{1/2}$ can be estimated from the slope of this plot. The steady state current i_{ss} is given as before, so by combining the slope of the plot given above with the steady state current a value of the concentration of the ferrocyanide, independent of the diffusion coefficient of the ferrocyanide in the cell can be estimated. This is given by:

$C = 2slope^2pi/(FALi_{ss})$ Eqn 5

In an example according to the preferred embodiments, a sample of blood is admitted to a thin layer cell containing a GOD/ferrocyanide system such as previously described with reference to Figures 12, 13, and 14. As illustrated in Figure 6, after allowing a short time **20** for reaction, an electric potential is applied between the electrodes, current flow commences when the potential is applied **21** but then falls as a transient **22** towards a steady state level **23**. The diffusion coefficient and/or glucose concentration are derived by measuring current as a function of time and by estimating the steady state current.

According to the preferred embodiments, the current is then interrupted, or reversed in polarity, for example by means of a suitable switch. If the polarity is reversed, a second transient is then observed, and a second steady state is reached after a further period of time although the profile is reversed. The underlying change in ferrocyanide concentration profile across the cell is shown schematically in Figure 7. The initial concentration profile prior to current reversal is **23**. The new steady state concentration profile is shown at **25**. The transient concentration profiles are exemplified at **24**.

By solving the diffusion equations for this situation, it can be shown that the transient current is described by.

$$\ln\left(\frac{i}{i_s} - 1\right) = \frac{-4\pi^2 Dt}{L^2} + \ln(4) \qquad \text{Eqn 6}$$

It is therefore simple to re-estimate the diffusion coefficient and concentration under the reversed polarity conditions. In theory the results should be independent of the type of transient or polarity. In practice, the results may differ due to factors affecting the transient such as sample inhomogeneity, state of the electrodes, or more importantly, due to asymmetries in the cell construction. This measure is therefore useful for cell diagnosis and also enables greater accuracy by allowing repetitive measurement and averaging with reverse polarities.

Similarly, if the potential is interrupted after steady state is reached, the initial concentration profile will be re-established in a short time (for example 4 seconds).
Once the initial state is re-established (or approximated) the potential can be re-applied and the procedure repeated without current reversal. Figure 9 shows a plot of current versus time similar to that of Figure 6 but having the potential interrupted at **26** and reapplied after 15 seconds at **27** yielding a new transient current **28** and then a state **29**.

As stated previously, the presence of oxygen in the blood is an interference since the concentration of final ferrocyanide is then not directly proportional to the initial glucose. Instead the initial glucose is related both to the final concentration of ferrocyanide plus hydrogen peroxide. However, it was found that hydrogen peroxide can be oxidised at the anode at a known potential which is higher than that for the ferrocyanide/ferricyanide redox reaction. The total electrochemical path is given in Figure 10. The hydrogen peroxide reaction is:

$$\text{hydrogen peroxide} \rightarrow \text{oxygen} + 2\,H^+ + 2\,e^- \qquad \text{reaction 7}$$

If, during the period of enzyme reaction a potential is applied (Figure 11) across the cell that is sufficient to oxidise hydrogen peroxide, then the following will happen during that period:
(a) glucose will be reacted to gluconic acid.
(b) ferrocyanide and hydrogen peroxide will result.
(c) the ferrocyanide/ferricyanide redox will eventually reach steady state.

(d) the peroxide will be oxidised at the anode and the electrons used to convert ferricyanide to ferrocyanide.

In total, after a period of time (approximately 2½ seconds in Figure 11) at a constant potential all the peroxide will be converted to oxygen (which is then a catalyst, and will return to complete more enzyme chemistry until glucose is exhausted), and the electrons utilised to convert ferricyanide to ferrocyanide.

At this stage (60 seconds in Figure 11) a reverse transient is applied. That is, the polarity of the cells is switched. but now at the lower potential suitable for the ferricyanide/ferrocyanide redox reaction. The final steady state ferrocyanide will once again reflect the initial glucose concentration. This can be analysed in the previously described manner to determine the total concentration of glucose in the initial sample.

Using the method of the preferred embodiments, the reaction phase of the test can be monitored in situ electrochemically without interfering with the measurement phase.

When the reaction is complete one can proceed to measurement without further delay.

The wait time will vary from test to test and will be the minimum necessary for any particular sample and cell, taking account of changes in enzyme activity from cell to cell as well as different temperatures and glucose concentrations. This is in stark contrast to prior art in which measurement is delayed until the maximum time required for reaction after allowing for all these factors.

In the present method the reaction phase is monitored by applying a potential between the two electrodes of, for example, -300 mV as soon as the cell begins to fill with sample.

A linear concentration profile of the reduced mediator is soon achieved across the cell. As more reduced mediator is produced by the enzyme reaction with glucose this linear concentration profile becomes steeper and the current increases. When the reaction is complete the current no longer increases. This point can be detected by well known electronic means and the measurement phase of the test can then be commenced.

The end-point of the reaction can also be estimated by fitting a theoretical kinetic equation to the current versus time curve generated during this part of the test.

This equation can predict the degree of completion of the reaction at any time. so would allow knowledge of when the end-point would occur without having to wait to get there.

This would further shorten the test time. For example, one could fit an equation to the measured prepulse current versus time curve. This equation could then predict that at time X the reaction will be, for example, 90% complete. If one measures the concentration at time X one would then divide the answer by 0.90 to get the true concentration.

The measurement of concentration in this system is done by reversing the potential, i.e. applying +300 mV between the electrodes. A current versus time curve will then occur, which is the same as that of the second transient in a double transient experiment le by transforming the current 1 measured during the measurement phase one can obtain a plot of $\ln(i/i_{ss} - 1)$ versus time which will have a slope of $-4pi^2D/l^2$ and an intercept ln(4). The normal analysis can then be used to obtain the concentration of glucose.

In some situations it may be difficult or impossible to know the distance between the electrodes in the electrochemical cell. For example, very small separations (ca. 10 microns) may be very difficult to manufacture or measure reproducibly. In these situations the use of information from two adjoining cells can be used to calculate the concentration of an analyte in a sample without knowledge of the cell separation if one of the cells contains a known concentration of the analyte or the corresponding reduced mediator prior to sample addition. Alternatively, a known quantity of this analyte or reduced mediator can be added to the sample destined for one of the two cells prior to addition of the sample to the cell. Another variation is if both cells contain a pre-determined analyte or reduced mediator concentration but each has a different concentration. Yet another variation is if two different predetermined quantities of the analyte or reduced mediator are added to two aliquots of the sample, which are then added to the adjoining cells.

The two electrochemical cells are then used in the normal fashion, and from each cell the following quantities are measured: steady state current (i_{ss}) and the slope of the straight line defined by $\ln(i/i_{ss} - 1)$ versus time, where i is the measured current. With a knowledge of these values and also a knowledge of the difference in concentration of the analyte or reduced mediator between the two cells, which is known (it is equal to that value purposely added to one cell), it is possible to calculate the concentration of analyte or reduced mediator in the sample, without any knowledge of the separation distance of the electrodes.

The above can be used in conjunction with a third cell that is used to measure the background current or concentration due to current caused by, for example, reduced mediator

formed by the application and drying of the chemistry, catalytic effect of the metal surface, oxidation of the metal surface, sample components that have effects on the analyte or mediator, electrochemically responsive components of the sample etc. This background concentration or current would be subtracted from the values measured from the two cells discussed above to calculate the true values for each cell resulting from the analyte in the sample, and in one case also the analyte or reduced mediator purposely added to the cell or the sample.

As will be apparent to those skilled in the art, from the teaching hereof the method is suitable for use with automatic measuring apparatus. Cells of the kind described may be provided with electrical connectors to an apparatus provide with a microprocessor or other programmed electronic control and display circuits which are adapted to make the required measurements perform the required calculations and to display the result. The method may be used to measure the concentration of analytes other than glucose and in liquids other than blood.

The method may be conducted using cells of other design and/or construction and using known catalysts and redox systems other than that exemplified.--

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